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ORIGINAL PAPER

Phylogenetic affiliation of the desert truffles *Picoa juniperi* and *Picoa lefebvrei*

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Abstract The molecular phylogeny and comparative morphological studies reported here provide evidence for the recognition of the genus *Picoa,* an hypogeous desert truffle, in the family Pyronemataceae (Ascomycota, Pezizales). *Picoa juniperi* and *Picoa lefebvrei* were reassigned to the genus *Picoa* based on large subunit (LSU) sequence (28S) rDNA and internal transcribed spacer (ITS) rDNA (including the partial 18S, ITS1, ITS2, 5.8S gene, and partial 28S of the nuclear rDNA) data. Morphological studies of spores, asci, perida, and gleba revealed high similarities between *P. lefebvrei* and *P. juniperi*, thereby confirming the membership of both species in the genus *Picoa*. These two species were primarily distinguishable based on ascospore ornamentation.

Keywords Pyronemataceae · *Picoa juniperi* · *Picoa lefebvrei* · Molecular phylogeny · ITS · LSU rDNA · Morphology

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Introduction

Picoa is hypogeous desert truffle (Ascomycetes) that has been documented in areas extending the Mediterranean to Middle East arid lands (Al-Scheikh and Trappe 1983; Moreno et al. 2000a, b; Ammarellou and Trappe 2007). It is presumed to establish a mutualistic association with roots of annual and perennial herbaceous plants of the *Helianthemum* genus (Gutiérrez et al. 2003; Slama et al. 2006). Five *Picoa* species are recorded in the Index Fungorum: *P. carthusiana*, *P. juniperi*, *P. lefebvrei*, *P. melospora*, and *P. pachyascus*. Unlike other truffles, the taxonomic status of the genus *Picoa* and the membership of the five proposed species in this genus remain uncertain, and several widely divergent taxonomic outlines have been reported. Vittadini (1831) was the first to propose the generic name *Picoa* and assign this truffle to the Tuberaceae family based on the type species *P. juniperi*. It was subsequently transferred from Tuberaceae to Terfeziaceae by Fischer (1897) and then to Balsamiaceae by Trappe (1979). *P. lefebvrei* was originally described as *Phaeangium lefebvrei* in 1894 by Patouillard who considered *P. lefebvrei* as the holotype species for the genus *Phaeangium*, but several other authors later considered this species to be member of the genus *Picoa* (Maire 1906; Moreno et al. 2000a, b). *P. carthusiana*, originally described by Tulasne and Tulasne (1862), was reassigned to *Leucangium carthusianum* by Trappe (1971) based on ascocarp differences with *P. juniperi*. Morphological

and molecular data have provided evidence for the membership of *L. carthusianum* within the Morchellaceae–Helvellaceae lineage (Li 1997; O'Donnell et al. 1997), while its exact assignment still remains uncertain (Læssøe and Hansen 2007). *P. pachyascus*, originally described by Lange (1956), was recently documented as a synonym of *Imaia gigantean* in Morchellaceae (Kovacs et al. 2008). *P. melospora* was described by Moreno et al. (2000a, b) from the Iberian Peninsula. This species, which has not yet been phylogenetically characterized, presents unusual morphological features for the genus, namely, one to five spores per ascus and elongated and smooth ascospores. Preliminary large subunit (LSU) rDNA sequence data suggest a close relationship between *P. juniperi* and *Otidea* spp. within the Pyrenomataceae family (O'Donnell et al. 1997). More recently, Tedersoo et al. (2010) considered *Picoa* to be a member of the *Geopora* lineage, but the exact phylogenetic positions of *P. juniperi* and *P. lefebvrei* have not yet been assessed.

In the study reported here, ascocarps morphologically characterized as those from *P. juniperi* and *P. lefebvrei*, respectively, were collected from the Tunisian arid lands. Based on sequence data on two genomic regions, we have confirmed that *Picoa* belongs to Pyronemataceae. Phylogenetic analyses revealed that this genus is closely related to *Geopora*.

Materials and methods

Origin of the samples

Fruit bodies were collected from the Medenine region in southern Tunisia (Table 1). This area is situated in a low-aridity bio-climatic zone with an average annual precipitation of 180 mm, a low average annual temperature of 19.9°C, and a mild winter. The average minimum temperature of the coldest month is 7°C, and the mean maximum temperature of the warmest months is 50°C.

The ascocarps were freshly harvested, superficially disinfected by shaking in 30% (v/v) H_2O_2 for 5 min. and aseptically rinsed several times with sterile water to eliminate any possibly trapped pocket of soil and microorganisms. The gleba was cut into small pieces kept at −80°C in sterile petri dishes before being freeze-dried overnight in a lyophilizator (FLEXI-Dry; FTS Systems, Milton, MA). Some samples were freshly used for microscopic observations. All samples have been deposited in the mycological specimen collection of the Royal Botanic Gardens Kew under the accession K(M)165772.

Morphological analysis

All fruit bodies were macro- and micro-morphologically characterized. Sections were mounted in 5%

Table 1 Collection of fruit bodies sequenced in this study

Species	Geographic origin	Host plant	IRA-MBA Herbarium accession[a]	GenBank LSU accession number	GenBank ITS accession number
Picoa juniperi	Medenine	*Helianthemum sessiliflorum*	IRA-MBAsb1	GU391549	GU391559
P. juniperi	Medenine	*H. sessiliflorum*	IRA-MBAsb2	GU391550	GU391560
P. juniperi	Medenine	*H. sessiliflorum*	IRA-MBAsb3	GU391551	GU391561
P. juniperi	Medenine	*H. sessiliflorum*	IRA-MBAsb4	GU391552	GU391562
P. lefebvrei	Medenine	*H. sessiliflorum*	IRA-MBAsb5	GU391553	GU391563
P. juniperi	Medenine	*H. sessiliflorum*	IRA-MBAsb6	GU391558	GU391564
P. juniperi	Medenine	*H. sessiliflorum*	IRA-MBAsb7	GU391554	GU391565
P. juniperi	Medenine	*H. sessiliflorum*	IRA-MBAsb8	GU391555	GU391566
P. juniperi	Medenine	*H. sessiliflorum*	IRA-MBAsb9	GU391556	GU391567
P. juniperi	Medenine	*H. sessiliflorum*	IRA-MBAsb10	ND	GU391568
P. lefebvrei	Medenine	*H. sessiliflorum*	IRA-MBAsb11	GU391548	GU391570
P. juniperi	Medenine	*H. sessiliflorum*	IRA-MBAsb12	GU391557	GU391569

[a] K(M)165772 is the accession in the mycological specimen collection of the Royal Botanic Gardens Kew

ITS, Internal transcribed spacer; LSU, large subunit; ND, not determined

KOH and cotton blue–lactophenol and observed under a light microscope at 1000× magnitude. KOH was not used for spore measurement because alkaline solutions may dissolve the spore ornaments (Ferdman et al. 2005). Cubes and slices, including the peridium and gleba, from fresh fruit bodies were embedded in paraffin, sectioned, and mounted for light microscopy. For scanning electron microscopy (SEM), dried material of the ascoma was dehydrated on a glass slide, then post fixed in osmium tetroxide, washed in phosphate buffer (pH 7.2), dehydrated first stepwise in ethanol (20–99%) and then in pure acetone, air-dried coated with gold–palladium, and examined using in a scanning electron microscope (Quanta 200; FEI, Hillsboro, OR).

DNA extraction, PCR amplification, and sequencing

DNA extraction was carried out on approximately 50 mg of freeze-dried fruit bodies. Tissues of the gleba were ground in liquid nitrogen. and nucleic acids were extracted according to the method of Henrion et al. (1994). DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 7.4; 1 mM of EDTA) and stored at −20°C. The internal transcribed spacer (ITS) and the 5′ LSU regions of the nuclear rDNA were separately amplified using the following primer pairs: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC- 3′) for the ITS rDNA; LROR (5′-ACCCGCTGAACTTAAGC-3′) and LR7 (5′-TACTACCACCAAGATCT-3′) for the 5′ LSU rDNA (Vilgalys and Hester 1990; White et al. 1990). The amplification reactions were performed in a 50-µl volume of reaction mixture [1 mM of each primer, 0.2 mM of each dNTP, and 2.5 U of *Taq* polymerase (Promega, Madison, WI] in a DNA thermal cycler (2400 geneAmp PCR thermocycler; Perkin Elmer, Foster City, CA). The cycling conditions were: an initial denaturation at 95°C for 2 min, followed by 35 cycles of a 1-min denaturation at 94°C, a 40-s annealing at either 53°C (ITS rDNA) or 47°C (LSU, 28S rDNA), and a 1-min elongation at 72°C, with a final elongation step at 72°C for 10 min. Amplification products were analyzed in 1.5% agarose gel in 0.5× TBE buffer (89 mmol l^{-1} Tris, 89 mmol l^{-1} borate, 2 mmol l^{-1} EDTA), stained with ethidium bromide, and visualized under UV light (Sambrook et al. 1989). The PCR products were purified with QIAquick Wizard PCR purification Kit (Promega) according to the manufacturer's instructions, and the sequences were determined by cycle sequencing using the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems; HTDS, Tunisia) and fragment separation in an ABI PrismTM 3130 DNA sequencer (Applied Biosystems; HTDS, Tunisia).

Sequence analysis

The 28S LSU and ITS rDNA nucleotide sequences were aligned using ClustalW (Thompson et al. 1997), and the alignment was manually edited with MEGA 4.0 (Tamura et al. 2007). Using RAxML (Stamatakis et al. 2005), we constructed a maximum-likelihood cladogram with 1000 fast bootstraps by following the GTR + G base substitution model using *Neolecta vittelina* and *Tarzetta catinus* sequences as the outgroup for the LSU and ITS analyses, respectively. The tree was edited with FigTree (Rambaut 2008). In parallel, a Bayesian inference was realized with MrBayes (Ronquist and Huelsenbeck 2003) using the GTR + G model and 1,000,000 generations. The sequences for *P. juniperi* and *P. lefebvrei* 28S LSU and ITS rDNA were submitted to GenBank under the accession numbers listed in Table 1.

Results

Morphological analysis

The *Picoa* fruiting bodies collected in this study (locally called "Zouber") appear in early February, when rainfall is adequate, at a soil depth close to 5 cm and near host plants (*H. sessiliflorum*). Fruiting bodies of *P. juniperi* were morphologically characterized (Fig. 1). The ascomata are 1–3 cm in size and very light in appearance; they have irregular forms and are often associated in clusters of four individuals. The peridium (Fig. 1a) has irregular pyramidal warts (and was more distinctly warty when dried) and is light brown to dark brown when young and blackish brown at maturity (Fig. 1b). The gleba is generally white, with fertile tissue separated by sterile veins (Fig. 1c). Asci (Fig. 1d and e) are of various shapes, double layered, hyaline, and thin walled and contain six to eight oval ascospores that are smooth

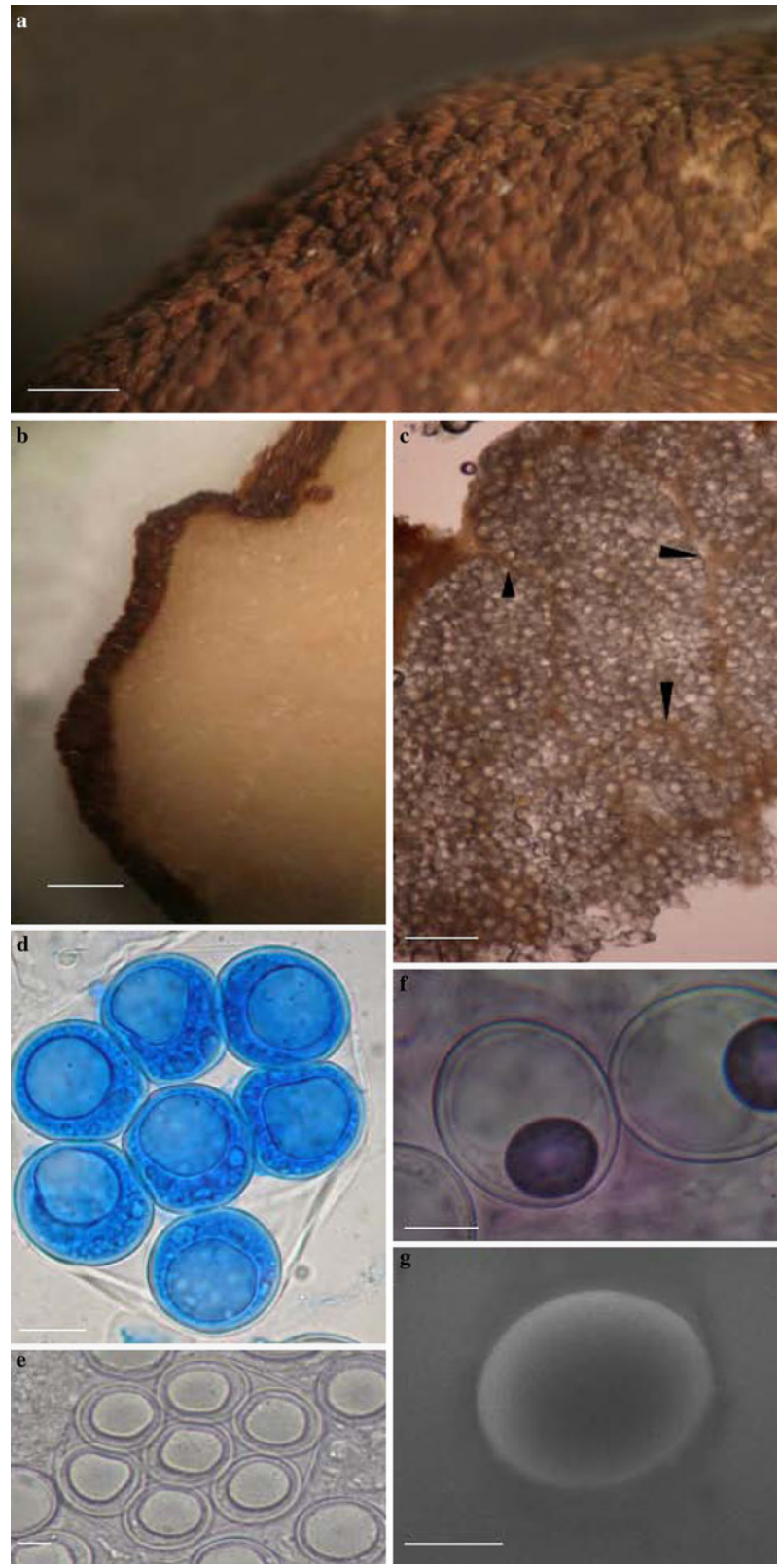

Fig. 1 Light and scanning electron micrographs of *Picoa juniperi* ascomata. **a** Warty peridium, *bar* 1 mm, **b** cross section of the gleba and peridium, *bar* 1 mm, **c** cross section of the gleba with sterile veins (*arrowheads*), *bar* 0.5 mm), **d** asci in cotton blue–lactophenol, *bar* 10 μm, **e** asci in 5% KOH, *bar* 10 μm, *f* mature ascospores showing a typical large lipid bodies (*l*), *bar* 10 μm, **g** smooth spore (in scanning electron microscopy), *bar* 10 μm. The white gleba appear *darker* in **c** due to the quality of the preparation (Color figure online)

Table 2 Characterization of *Picoa*, *Leucangium*, and *Geopora* genera

Characteristics	*Picoa juniperi*	*Picoa lefebvrei*[a]	*Leucangium carthusianum*[b]	*Geopora* sp[c]
Ascoma	Hypogeous with irregular forms, stereothecia	Hypogeous, gregarious, sub-globose, stereothecia	Hypogeous, stereothecia	Epigeous, several species have a small subterranean apothecia, ptychothecia[e]
Peridium	Warty, brown to dark brown	Reddish brown to dark brown with irregular pyramidal rounded warts.	Dark with medullary excipulum with cylindrical to isodiametric cells	Outer surface irregular to warted or furrowed, covered with dark hairs
Gleba	White, crumbly, with fertile pockets separated by sterile vein clearly distinguished	Off-white, very crumbly, with fertile pockets separated by sterile veins.	Dark, very crumbly with fertile pockets separated by sterile veins	None
Spore shape	Oval	Oval	Lemon shaped	Subglobose, ellipsoid
Spore ornamentation	Smooth	Warty	Smooth	Smooth
Host plant	*Cistaceae Helianthemum sessiliflorum*[d]	*Cistaceae Helianthemum* sp.[d]	*Pseudotsuga menziesii*	Wide range of hosts Pinaceae[d]

[a] According to Patouillard (1894), Maire(1906), Moreno et al. (2000a, b), and Gutiérrez et al. (2003)

[b] According to Patouillard (1894), Maire (1906), Li (1997), and Palfner and Agerer (1998)

[c] According to Harold and Burdsall (1965, 1968); Jack and Gaud (1997). and Wei et al. (2010)

[d] Preferred host plant

[e] Except for *Geopora cooperi* and *G. clausa*. Læssøe and Hansen 2007; Smith and Healy 2009

and contain a dark lipid body at maturity (Fig. 1f, g). Morphological features of *P. juniperi*, *P. lefebvrei*, and *L. carthusianum* are listed in Table 2.

Phylogenetic analysis

Analysis of the 5′ end of the LSU rDNA region (including the D1 and D2 domains) data set of 36 Pezizalean fungi, including the 11 specimens (from IRA-MBAsb1 to IRA-MBAsb11) sequenced in this, was performed using maximum-likelihood and Bayesian methods. The topologies of the phylogenetic trees built with maximum likelihood and Bayesian inference were similar and clearly indicate that *Picoa* is a member of the Pyronemataeae. The 11 *Picoa* specimens, which share 95–99% sequence identity and form a coherent cluster well supported by significant bootstrap and posterior probability values (86–100%), are most closely related to *Geopora* species (IRA-MBAsb11 shows the highest sequence identity; 97% with *Geopora cooperi* DQ220342). Notwithstanding, *Geopora* spp. differ from *Picoa* spp. on the basis of the development mode, forms, spore discharge, and the associative host plant (Table 2). Noticeably, *Leucangium carthusianum* (synonym *Picoa carthusiana*) was reliably placed in the Morchellaceae lineage (78% similarity in base pairs) and shares 95% LSU sequence identity with *Imaia gigantea* (synonym of *Picoa pachyascus*) (Fig. 2).

These results were further tested and confirmed based on the phylogeny inferred from the analysis of the ITS rDNA sequences. Moreover, the generated phylogenetic tree (Fig. 3) supports the separation of the genus *Picoa* into two clusters with significant bootstrap and posterior probability values (92–100%): (1) cluster one associating *P. juniperi* specimens and (2) cluster two regrouping *P. lefebvrei* specimens.

Discussion

The microscopic comparisons performed in this study and in previous studies reveal that there are distinct morphological links between *P. juniperi* and *Phaeangium lefebvrei* that can allow them to be recognized as members of the same genus. The main difference between these two species is the ascospore ornamentation. Using specimens collected in Kuwait,

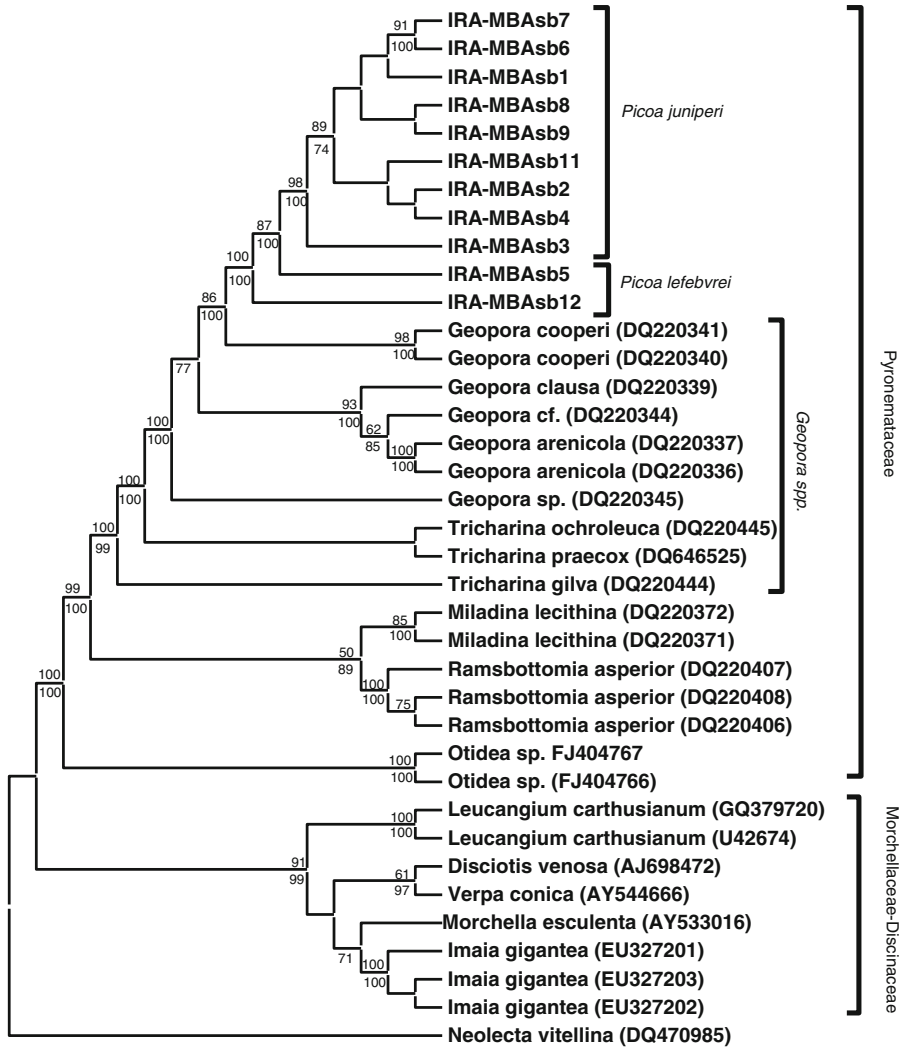

Fig. 2 Maximum-likelihood cladogram inferred from the 787-bp large subunit (LSU) region alignment, demonstrating the placement of *Picoa* within *Pyrenomataceae*. Bootstrap values >50 are shown *above* branches, and posterior probability values >50 are shown *below* branches. The phylogenetic trees were built with maximum likelihood and Bayesian inferences are topologically similar

Iraq, North Africa (Al-Scheikh and Trappe 1983), and Tunisia (data not shown), we have shown that *P. juniperi* spores are oval and smooth while those of *P. lefebrei* appear warty and ornamented at maturity.

The molecular positioning of *L. carthusianum* (syn. *Picoa carthusiana*) within the Morchellaceae–Discinaceae lineage, proposed in this study, corroborates earlier morphological and ecological data and confirms the exclusion of this species from the genus *Picoa* (O'Donnell et al. 1997). An ultra-structural study of *L. carthusianum* performed by Li (1997) showed that this species is characterized by lemon-shaped and multinucleated ascospores. This species has been reported in Europe and North America (Trappe 1971) in mutualistic association with the forest tree *Pseudotsuga menziesii* (Palfner and Agerer 1998).

Ribosomal DNA analyses have enabled the genus *Picoa* to be assigned to the Pyronemataceae and to confirm that *Picoa* is closely related to *Geopora* (Tedersoo et al. 2010). The phylogenetic position of *P. juniperi* and *P. lefebrei* proposed in this report is well supported by morphological and ecological features. The two *Picoa* species are hypogeous taxa

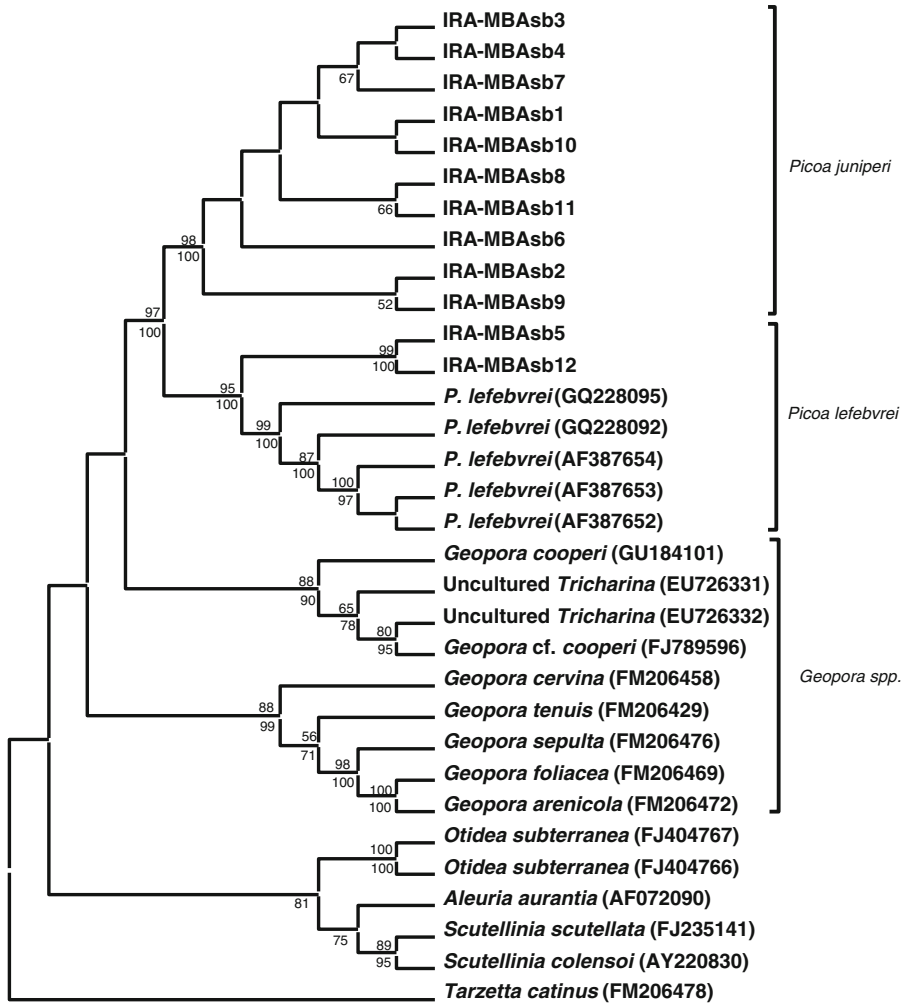

Fig. 3 Maximum-likelihood cladogram based on the 413-bp internal transcribed spacer (ITS) region alignment of *Picoa*, *Geopora*, and related *Pyrenomataceae* species. Bootstrap values >50 are shown *above* branches, and posterior probability values >50 are shown *below* branches. Topologies of the phylogenetic tree with maximum likelihood and Bayesian inferences are similar

with white to off-white and crumbly gleba (stereothecia) and the absence of forcible spore discharge. *Geopora* species possess hollow ascocarps (ptychothecia), are epigeous or partially hypogeous (hypogeous in their early stages of development, except for *Geopora cooperi* and *G. clausa*, which are mostly hypogeous; Læssøe and Hansen 2007; Smith and Healy 2009), and emerge at the ground surface at maturity with few (if any) convolutions and a functional operculum that opens at the ground surface (Harold and Burdsall 1965, 1968). The ascospores of these *Geopora* species are discharged through an operculum. *Picoa* species are also divergent from *Geopora* spp. based on the host plants and the geographic distribution. *P. juniperi* and *P. lefebrei* occur in the Mediterranean region and in Middle East arid lands, and they are associated with members of *Cistaceae* and, preferentially, with *Helianthemum* species (Moreno et al. 2000a, b; Gutiérrez et al. 2003; Slama et al. 2006). In contrast, *Geopora* spp. are associated with a wide range of host plants that are essentially found in Pinaceae forest stands (Harold and Burdsall 1965, 1968; Jack and Gaud 1997; Wei et al. 2010).

Based on the results of our study, we conclude that the genus *Picoa* is a close relative of *Geopora* within the family Pyronemataceae. *P. juniperi* and *P. lefebvrei*, the two recognized species of the genus,

form together with *Otidea subterranea* (Smith and Healy 2009) the only known hypogeous and mycorrhizal truffles with a stereothecia in the family Pyronemataceae.

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